Atty. Dkt. No. 034827-2301

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Sun, et al.

Title: METHODS AND COMPOSITIONS FOR THE DETECTION OF MUCOLIPIDOSIS IV MUTATIONS

Appl. No.: 10/754,446

Filing Date: 1/9/2004

Examiner: Kapushoc, Stephen Thomas

Art Unit: 1634

Confirmation Number: 7990

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APPEAL BRIEF TRANSMITTAL

Mail Stop Appeal Brief-Patents
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Sir:

Transmitted herewith is an amendment in the above-identified application.

[X] Appeal Brief (19 pages including 3 appendices, and 5 exhibits).

[X] Credit Card Payment Form.

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[X] The fee required for additional claims is calculated below:

	Claims As Amended		Previously Paid For		Extra Claims Present		Rate		Additional Claims Fee
Total Claims:	16	-	36	=	0	x	$50.00	=	$0.00
Independent Claims:	2	-	5	=	0	x	$200.00	=	$0.00
First presentation of any Multiple Dependent Claims:						+	$360.00	=	$0.00
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[ ] Applicant hereby petitions for an extension of time under 37 C.F.R. §1.136(a) for the total number of months checked below:

[X]	Appeal Brief Fee	$500.00
	FEE TOTAL:	$500.00
[X]	Small Entity Fees Apply (subtract ½ of above):	$0.00
	TOTAL FEE:	$500.00

If any extensions of time are needed for timely acceptance of papers submitted herewith, applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 50-0872.

Please direct all correspondence to the undersigned attorney or agent at the address indicated below.

Respectfully submitted,

Date 05/11/2007

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05-14-07

AF/
IFW

PATENT
034827-2301

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Sun et al.
Title:	METHODS AND COMPOSITIONS FOR THE DETECTION OF MUCOLIPIDOSIS IV MUTATIONS
Appl. No.:	10/754,446
Filing Date:	January 9, 2004
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APPEAL BRIEF

Mail Stop Appeal Brief - Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Applicants (hereinafter "Appellants") hereby appeals the Final Rejection of claims 19-34, which corresponds to all claims pending in the application. This Appeal Brief follows a Notice of Appeal which was filed on March 12, 2007 and is accompanied by the requisite fee set forth in 37 C.F.R. § 1.17(f). If this fee is incorrect or if any additional fees are due in this regard, please charge or credit our Deposit Account No. 50-0872 for the appropriate amount.

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CASES

RULES

Real Party in Interest

The real party in interest in this appeal is Quest Diagnostics Incorporated, which is the assignee of the present application.

Related Appeals and Interferences

None.

Status of Claims

Claims 1-18 and 35-36 have been cancelled.

Claims 19-34 are pending in the application.

Claims 19-34 are the subject of this appeal.

Status of Amendments

The last claim amendments were presented in Appellants' Response of August 10, 2006. The claim amendments contained therein have been entered, examined, and appealed herein. No other amendments or submissions are pending in the application.

Summary of Claimed Subject Matter

Mucolipidosis type IV (MLIV) is a lyposomal storage disorder that affects the nervous system.[1] MLIV is an inherited disease and the majority of MLIV patients never develop the ability to speak or walk and remain at a developmental age of 1-2 years.[2] It is known that MLIV

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[1] Specification at ¶¶ 3-4 and 7.

[2] Id. at ¶ 6.

is caused by a mutation in the MCOLN1 gene which encodes the mucolipin-1 membrane protein.[3]

Two different MCOLN1 mutations have been identified; a deletion mutation and a polymorphism mutation. The deletion mutant is characterized by a 6.4 kb deletion that includes exons 1-7.[4] The polymorphism mutant (IVS 3-2 A>G) is characterized by an A-to-G transition which causes a splice site mutation in the acceptor site of Intron 3 (hereinafter referred to as "the point mutation").[5] Because the site of the point mutation is contained within the genomic region deleted in the deletion mutation, only three allelic variants exist: (i) wt (no deletion, no point mutation), (ii) A>G (no deletion, point mutation present), and (iii) del (deletion mutation). The claimed subject matter relates to real-time PCR methods for detecting these mutations.

Support for claim 19 is found in the specification at ¶ 14.

Support for claims 20-22 is found in the specification at ¶¶ 10-11.

Support for claim 25 is found in the specification at ¶ 15.

Support for claims 26-31 and 34 is found in the specification at ¶¶ 10-11 and 16.

Grounds for Rejection to be Reviewed on Appeal

1. Claims 19 and 23-24 stand finally rejected under 35 U.S.C. § 103 as allegedly obvious over Edelmann et al. (Am. J. Hum. Genet. 70: 1023-1027, 2002), as evidenced by GenBank AF287270 (2000), in view of Doll et al. (Anal. Biochem. 301: 328-332, 2002).

2. Claims 25, 28, and 32-34 stand finally rejected under 35 U.S.C. § 103 as allegedly obvious over Edelmann et al. (Am. J. Hum. Genet. 70: 1023-1027, 2002), as evidenced by GenBank AF287270 (2000), in view of Doll et al. (Anal. Biochem. 301: 328-332, 2002).

3. Claims 20-22, 26-27, and 29-31 stand finally rejected under 35 U.S.C. § 103 as allegedly obvious over Edelmann et al. (Am. J. Hum. Genet. 70: 1023-1027, 2002) in view of

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[3] Id. at ¶ 5.

[4] Id. at ¶ 4.

[5] Id.

Doll et al. (Anal. Biochem. 301: 328-332, 2002), GenBank AF287270 (2000), and Buck et al. (Biotechniques 27: 528-536, 1999).

Argument

1. Rejection of claims 19 and 23-24 under 35 U.S.C. § 103 over Edelmann et al., as evidenced by GenBank AF287270, in view of Doll et al.

1.1. The legal standard for obviousness.

In order to make a *prima facie* case of obviousness, the Examiner must demonstrate that the prior art (i) teaches or suggests every claim limitation, (ii) provides a motivation to combine (or modify) the teachings of the selected references, and (iii) provides a reasonable expectation of success. In re Vaeck, 947 F.2d 488, 493, 20 USPQ2d 1438 (CAFC 1991); MPEP § 2143. This is the "TSM" test for obviousness which was recently affirmed by the Supreme Court. KSR Int'l Co. v. Teleflex Inc., No. 04-1350, 550 U.S. ____, slip op. at 15 (2007). In explicating the correct standard for this test, the KSR Court reaffirmed previous holdings that an invention "is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art." KSR, slip op. at 14.; see also, In re Rouffet, 149 F.3d 1350, 1357, 47 USPQ2d 1453, 1457 (Fed. Cir. 1998). Furthermore, the Court warned the factfinder to be aware of the distortion caused by hindsight bias and to be cautious of arguments reliant upon *ex post* reasoning. KSR, slip op. at 17.

1.2. Appellants' claimed invention.

Claim 19 provides methods for real-time detection the MLIV deletion mutation (i.e., the 6.4 kb deletion that includes exons 1-7 of the MCOLN1 gene). Oligonucleotide primers (e.g., SEQ ID NOs.: 3 and 4; "PRI primers") which bind to nucleotides 100-500 and 6956-7356 of the MCOLN1 gene (SEQ ID NO: 8) are used for amplification.[6] These flanking primers result in an amplicon from the deletion mutant that is, at most, 800 nucleotides in length, but may be shorter based on specific primer selection. The amplicon, if present, is detected using a polynucleotide

[6] Specification at ¶¶ 36 and 50.

probe (e.g., SEQ ID NO.: 7) which is labeled with a donor fluorophore and an acceptor fluorophore (i.e., a quencher).[7] When unbound, the donor and acceptor fluorophores are in close proximity so little or no fluorescent signal from the donor fluorophore is detected. Upon binding of the labeled probe to the PCR product, the donor and acceptor fluorophores become spatially separated and the fluorescent signal from the donor fluorophore is increased.[8] In one variation of the claimed method, the PCR reaction is performed in the presence of a polymerase with 5' exonuclease activity which cleaves the double-stranded product formed by the hybridization of the probe and the PCR product.[9] This ensures permanent separation of the donor and acceptor fluorophores resulting in a robust and long-lasting increase in detectable fluorescence from the donor fluorophore.

1.3. Edelmann et al. and Doll et al. fail to teach or suggest every element of the claimed method.

The combination of Edelmann et al. and Doll et al. fails to teach every element in Appellants' method of claim 19. Edelmann et al. disclose a multi-step method for detecting MCOLN1 gene mutations. Edelmann et al. first perform a multiplex PCR amplification that results in one amplicon from the deletion mutant, if present, and a second amplicon encompassing the site of the point mutation. The PCR product(s) was immobilized and probed with radio-labeled allele-specific oligonucleotides for the point mutation, its wildtype, and the deletion mutation.[10] Thus, as acknowledged by the Examiner, Edelmann et al. fail to disclose genotyping multiple mutations (including the deletion mutation) of the MCOLN1 gene using real-time PCR.[11]

Doll et al. does not provide what Edelmann et al. lack. Doll et al. provide a method for detecting single nucleotide polymorphisms (i.e., point mutations) using fluorogenically-labeled probes. But, Doll et al. do not teach or suggest a method for detecting deletion mutations, as required by claim 19. Doll et al. recognized the existence of deletion mutations in the gene of

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[7] Id. at ¶¶ 11 and 16-18.

[8] Id. at ¶ 45.

[9] Id. at ¶¶ 46-47 and claims 24 and 33.

[10] Edelmann et al. at page 1024, right column, first paragraph.

[11] Final Office Action mailed October 11, 2006, at page 6, first paragraph.

interest (NAT1).[12] However, despite this recognition, Doll et al. do not present a method for their detection. Thus, the Examiner has purely and improperly relied on a hindsight analysis, using nothing more than Appellants' specification, to "adapt" the method of Doll et al. for the detection of a deletion mutation.

Neither Edelmann et al. nor Doll et al. teach or suggest a method for the real-time detection of a <u>deletion</u> mutation in the MCOLN1 gene as required by claim 19. Because the combination of prior art asserted by the Examiner fails to teach every element of claim 19, this rejection should be reversed and withdrawn.

<u>1.4. There is no motivation to combine the teachings of Edelmann et al. and Doll et al.</u>

The Examiner fails to demonstrate that the prior art provides a motivation to combine the methods of Edelmann et al. and Doll et al. in an attempt to derive the method of claim 19. Edelmann et al. are concerned with detecting two different types of mutations in the MCOLN1 gene; a point mutation and a deletion mutation. Edelmann et al. use a method in which the genomic regions of interest are amplified by PCR. The PCR product is then probed, in a second step, using radio-labeled allele-specific oligonucleotides.

There exists no motivation to combine the teachings of Doll et al. with Edelmann et al. Each of the mutations detected by Doll et al. are point mutations.[13] Doll et al. are not concerned with detecting <u>deletion</u> mutations despite the fact that Doll et al. recognize their existence as alternate NAT1 alleles (the gene of interest to Doll et al.).[14] The Examiner has failed to identify a reason why a skilled artisan, understanding the method of Edelman et al., would be motivated to modify the portion of that method which relates to detecting deletion mutations with anything taught by Doll et al. which is directed solely to the detection of point mutations.

In sum, the combination of Doll et al. and Edelmann et al. fail to teach or suggest the use of allele-specific oligonucleotides to detect deletion mutations, as required in claim 19. Furthermore, there exists no motivation to combine the methods of Doll et al. and Edelmann et al. because Doll et al. are concerned with mutations in a gene unrelated to the MCOLN1 gene

[12] <u>Doll et al</u>. at Table 1.

[13] <u>Id</u>. at Table 2 and page 331, first paragraph.

[14] <u>Id.</u> at Table 1.

and, the mutations detected by Doll et al. are point mutations only. Appellants' respectfully submit that the Examiner has failed to make a *prima facie* case of obviousness and request that this rejection be reversed and withdrawn.

2. Rejection of claims 25, 28 and 32-38 under 35 U.S.C. § 103 over Edelmann et al., as evidenced by GenBank AF287270, in view of Doll et al.

Claims 28 and 32-38 each depend from claim 25. The method of claim 25 may be used to simultaneously detect the deletion mutation and the point mutation of the MCOLN1 gene. The deletion mutation is detected using substantially the same method as described above and encompassed by claim 19. The point mutation is detected by the further addition of PCR primers (e.g., SEQ ID NOs.: 1 and 2) which flank the mutation site.[15] The presence of the point mutation is determined using fluorophore-labeled probe specific for that sequence (e.g., SEQ ID NO.: 6). Optionally, a fluorophore-labeled probe that is specific for the wildtype sequence (e.g., SEQ ID NO.: 5) may be used in addition to, or instead of the mutant-specific probe.

Appellants submit that, for the reasons provided above, the method of claim 19 is unobvious. The methods of claims 25, 28, and 32-38 encompass all of the limitations of claim 19 and are, therefore, also unobvious for the same reasons. Additional arguments for the patentability of claims 25, 28, and 32-38 follow.

2.1. Edelmann et al. and Doll et al. fail to teach or suggest every element of the claimed method.

Claim 25 encompasses a method for the real-time detection of the MCOLN1 deletion mutation and the point mutation. The method requires a multiplex PCR using a primer pair which flanks the deleted region and another primer pair which flanks the site of the point mutation. The two MCOLN1 mutations are necessarily represented on different amplicons and are detected in real-time using allele-specific oligonucleotides containing fluorescent labels.

For the reasons discussed in Section 1.3 (*supra*), Appellants reassert that the combination of Edelmann et al. and Doll et al. fail to teach every element in the method of claim 25 insofar as

[15] Specification at ¶¶ 13-14.

claim 25 encompasses the real-time detection of the deletion mutation of the MCOLN1 gene. Specifically, Edelmann et al. detect MCOLN1 mutations, including a deletion mutation, using a two-part method which cannot be used in a real-time PCR-based genotyping assay. Doll et al. do not remedy the deficiency of Edelmann et al. because Doll et al. do not detect any deletion mutation, let alone the simultaneous detection of a deletion mutation and a point mutation. Thus, the combination of Edelmann et al. and Doll et al. fail to teach every element of claim 25.

2.2. There is no reasonable expectation of success for combining the methods of Edelmann et al. and Doll et al.

Also missing from the combination of the methods of Edelmann et al. and Doll et al. is a teaching of how to simultaneously detect multiple mutations on multiple amplicons in real-time. The method of claim 25 results in the production of two distinct amplicons. The first amplicon, if present, encompasses the region of Intron 3 that contains the A>G transition site and is produced from the first and second oligonucleotide primers. The second amplicon, if present, is produced from the third and fourth oligonucleotide primers and is indicative of the presence of the MCOLN1 deletion mutant.

Edelmann et al. detect the multiple MCOLN1 gene mutations on multiple amplicons but, this method cannot be adapted for real-time detection. Edelmann et al. immobilize the amplification products on a solid support and then detect those products with a radio-labeled oligonucleotide probe.

This deficiency is not remedied by Doll et al. The method of Doll et al. detects multiple mutations on the a single amplicon. Doll et al. provide no guidance on how the method might be adapted for use with multiple PCR primers resulting in multiple amplicons. Thus, the combination Edelmann et al. and Doll et al. fails to teach, and provides no reasonable expectation that multiple types of mutations (i.e., point mutations and deletion mutations) can be simultaneously detected on multiple amplicons in a real-time PCR-based assay as recited in claim 25.

In sum, the rejection of claims 25, 28 and 32-38 is flawed in several respects. The combination of Edelmann et al. and Doll et al. fail to teach a method for the real-time detection of a deletion mutation in any gene and they specifically fail to teach a method for the real-time detection of the MCOLN1 deletion mutation. Furthermore, the combination of Edelmann et al.

and Doll et al. does not provide the artisan with a reasonable expectation of success because this combination does not demonstrate how to detect multiple type of mutations (deletion mutations and point mutations) on multiple amplicons in real-time. Therefore, Appellants respectfully submit that this rejection should be reversed and withdrawn.

3. Rejection of claims 20-22, 26-27 and 29-31 under 35 U.S.C. § 103 over Edelmann et al., in view of Doll et al., GenBank AF287270 and Buck et al.

Claims 20-22 depend from claim 19 and claims 26-27 and 29-31 depend from claim 25. Each of the reasons discussed above for the unobviousness of claims 19 and 25 are reasserted with respect to claim 20-22, 26-27 and 29-31. Appellants submit that those reasons alone or in combination are sufficient to prove these claims are also unobvious. Additional arguments for the patentability of claims 20-22, 26-27 and 29-31 follow.

3.1. The prior art does not teach the specific primers and probes recited in the rejected claims.

Claims 20-21 and claims 29-30 require the use of the oligonucleotide primers of SEQ ID NOs.: 3 and 4 for amplification of the deletion mutation of the MCOLN1 gene. Claims 26-27 require the use of oligonucleotide primers of SEQ ID NOs.: 1 and 2 for amplification of the point mutation of the MCOLN1 gene. Claims 23 and 31 require the use of oligonucleotide probe of SEQ ID NO.: 7 for detection of the deletion mutation of the MCOLN1 gene. The Examiner acknowledges that the prior art does not teach any of these specific oligonucleotide primers or probes.[16]

In an attempt to remedy this deficiency in the prior art, the Examiner points to GenBank AF287270 which provides the nucleic acid sequence of the MCOLN1 gene and that Buck et al. demonstrate the equivalence of primers. The Examiner alleges that this combination renders obvious the use of any primer based on any known nucleic acid sequence.[17] Appellants strongly disagree.

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[16] Final Office Action mailed October 11, 2006 at page 12, second paragraph.

[17] Id. at page 13.

As an initial matter, with respect to claims 23 and 31 which relate to the use of the oligonucleotide probe of SEQ ID NO.: 7, Appellants point out that the Examiner has failed to provide any reasons why the use of this probe, which is not disclosed in the prior art, is obvious. Notwithstanding this oversight, the teachings of Buck et al. are not concerned with oligonucleotide probes and are, therefore, irrelevant to this rejection insofar as the rejection relates to the specific probes of claims 23 and 31.

Turning next to the issue of primers, Buck et al. demonstrate the equivalence of <u>sequencing</u> primers. Specifically, Buck et al. perform a sequencing reaction using every primer spaced at a three nucleotide interval over a 300 nucleotide sequence and demonstrate that virtually every primer was successful. Appellants emphasize that Buck et al. are concerned with testing the effectiveness of <u>sequencing</u> primers which are used individually for a DNA polymerase reaction that is randomly terminated prior to the completion of a full copy of the target DNA. By contrast, PCR primers are used in a reaction that creates a full copy of the target DNA in which the primers are used in pairs to define the boundaries of the target DNA.

Appellants further urge that Buck et al. do not render obvious the use of any MCOLN1 primers in the present invention because Buck et al. are not concerned with real-time amplification assays. As attested by Dr. Sun, the presently clamed invention requires the use of PCR primers and hybridization probes in a real-time format (e.g., a Taq-man assay) which is more complex than standard assays.[18] The probes and primers must be capable of working together because the probes must bind to the amplicons, under the PCR conditions, as the amplicons are generated. Dr. Sun concludes that there is no expectation that any set of PCR primers, combined with any oligonucleotide hybridization probe will be successful in a real-time amplification assay.[19]

Thus, the sequencing primers of Doll et al. cannot be equated with PCR primers generally, or with multiplex primers used in a real-time amplification assay format as required by the rejected claims. Appellants respectfully submit that is rejection is traversed and should be withdrawn.

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[18] Declaration of Weiman Sun, Ph.D. under 37 CFR § 1.132, filed August 10, 2006.

[19] <u>Id</u>.

Conclusion

For the reasons discussed above, Appellants respectfully submit that claims 19-34 are in condition for allowance, and respectfully request that the rejections be withdrawn or reversed, and that the claims be allowed to issue.

Respectfully submitted,

Date: 05/11/2007

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Appendix A: Claims Appendix

1-18. (Cancelled)

19. (Previously Presented) A method of determining the presence of a Mucolipidosis IV deletion mutation sequence in a nucleic acid, comprising,

a) contacting the nucleic acid with:
   i) a first oligonucleotide primer that comprises a sequence complementary to a 15-30 bp segment of DNA between positions 100-500 of the MCOLN1 gene (SEQ ID NO: 8),
   ii) a second oligonucleotide primer that comprises a sequence complementary to a 15-30 bp segment of DNA between positions 6956-7356 of the MCOLN1 gene (SEQ ID NO: 8), and
   iii) an oligonucleotide probe that comprises a sequence complementary to a 13-30 bp segment of a fragment that is amplified using the first and second primer, wherein said probe is labeled with a detectable label which comprises a donor fluorophore and a quencher moiety, wherein said quencher moiety is optionally an acceptor fluorophore; and
b) conducting amplification by temperature cycling and monitoring the accumulation of amplified nucleic in real time by detecting an increase in donor fluorophore fluorescence or a decrease in acceptor fluorophore fluorescence which indicates the presence of the Mucolipidosis IV mutant sequence in the nucleic acid.

20. (Original) The method of claim 19 wherein the first oligonucleotide primer comprises a sequence that consists essentially of 5'-CTT GCT CTG TTG CCC AGG CT -3' (SEQ ID NO. 3).

21. (Currently amended) The method of claim 19 wherein the second oligonucleotide primer comprises a sequence that consists essentially of 5'-CTC ACC GTG CTG GAA GAC ACT -3' (SEQ ID NO. 4).

22. (Previously Presented) The method of claim 19 wherein the probe comprises a sequence that consists essentially of 5'- AGACC CAG GCC CAC AT- 3' (SEQ ID NO: 7).

23. (Original) The method of claim 19 wherein the donor fluorophore is 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC), 6-carboxyfluorescein (FAM) or tetrachloro-6-carboxyfluorescein (TET).

24. (Original) The method of claim 19 wherein amplification by temperature cycling is with a DNA polymerase with 5' exonuclease activity and wherein binding of the probe to amplified nucleic acid results in degradation of the probe during DNA synthesis.

25. (Previously Presented) A method of detecting the presence of one or two Mucolipidosis IV mutant sequences in a nucleic acid, comprising,

a) contacting the nucleic acid with:
   i) a first oligonucleotide primer that comprises a sequence complementary to a 15-30 bp segment of DNA between positions 5124-5524 of the MCOLN1 gene (SEQ ID NO: 8),
   ii) a second oligonucleotide primer that comprises a sequence complementary to a 15-30 bp segment of DNA between positions 5541-5941 of the MCOLN1 gene (SEQ ID NO: 8),
   iii) a first oligonucleotide probe that comprises a sequence complementary to a 13-30 bp segment of a fragment that is amplified using the first and second oligonucleotide primers, wherein said first oligonucleotide probe includes position 5534 of the MCOLN1 gene (SEQ ID NO: 8), wherein said first oligonucleotide probe is labeled with a first detectable label which comprises a donor fluorophore and a quencher moiety, wherein said quencher moiety is optionally an acceptor fluorophore:
   iv) a third oligonucleotide primer that comprises a sequence complementary to a 15-30 bp segment of DNA between positions 100-500 of the MCOLN1 gene (SEQ ID NO: 8),
   v) a fourth oligonucleotide primer that comprises a sequence complementary to a 15-30 bp segment of DNA between positions 6956-7356 of the MCOLN1 gene (SEQ ID NO: 8), and

vi) a second oligonucleotide probe that comprises a sequence complementary to a 13-30 bp segment of a fragment that is amplified using the third and fourth primers, wherein said probe wherein said second oligonucleotide probe is labeled with a second detectable label which comprises a donor fluorophore and a quencher moiety, wherein said quencher moiety is optionally an acceptor fluorophore, and wherein said second detectable label is distinguishable from said first detectable label; and

b) conducting amplification by temperature cycling and monitoring the accumulation of amplified nucleic in real time by detecting an increase in donor fluorophore fluorescence or an increase or decrease in acceptor fluorophore fluorescence, which indicates the presence of one or both of the Mucolipidosis IV mutant sequences in the nucleic acid, wherein said first and second primer and first probe detect a single base transition mutation and said third and fourth primer and second probe detect a deletion mutation.

26. (Original) The method of claim 25 wherein the first oligonucleotide primer comprises a sequence that consists essentially of 5'-AGC GGG CCG GAC TCA-3' (SEQ ID NO. 1).

27. (Original) The method of claim 25 wherein the second oligonucleotide primer comprises a sequence that consists essentially of 5'-TAA CCA CCA TCG GAT CAA TGT C-3' (SEQ ID NO. 2).

28. (Original) The method of claim 25 wherein the first probe comprises a sequence that consists essentially of 5'- CTGC CCA CGG TAC CT -3' (SEQ ID NO: 6).

29. (Original) The method of claim 25 wherein the third oligonucleotide primer comprises a sequence that consists essentially of 5'-CTT GCT CTG TTG CCC AGG CT -3' (SEQ ID NO. 3).

30. (Original) The method of claim 25 wherein the fourth oligonucleotide primer comprises a sequence that consists essentially of 5'-CTC ACC GTG CTG GAA GAC ACT -3' (SEQ ID NO. 4).

31. (Original) The method of claim 25 wherein the second probe comprises a sequence that consists essentially of 5'- AGACC CAG GCC CAC AT- 3' (SEQ ID NO: 7).

32. (Original) The method of claim 25 wherein the first or second donor fluorophore is 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC), 6-carboxyfluorescein (FAM) or tetrachloro-6-carboxyfluorescein (TET).

33. (Original) The method of claim 25 wherein amplification by temperature cycling is with a DNA polymerase with 5' exonuclease activity and wherein binding of the probe to amplified nucleic acid results in degradation of the probe during DNA synthesis

34. (Original) The method of claim 25 wherein said nucleic acid containing sample is also contacted with a third oligonucleotide probe comprising a sequence consisting essentially of 5'- TCTG CCC ACA GTA CCT -3' (SEQ ID NO: 5) that hybridizes to a wildtype sequence, wherein said third probe is labeled with a detectable label comprising a donor fluorophore and a quencher moiety wherein said quencher moiety is optionally an acceptor fluorophore, and wherein said third detectable label is distinguishable from said first and second detectable labels.

35-36. (Cancelled)

Appendix B: Evidence Appendix

1. Buck et al. Biotechniques 27: 528-536, 1999.

2. Doll et al. Anal. Biochem. 301: 328-332, 2002.

3. Edelmann et al., Am. J. Hum. Genet. 70: 1023-1027, 2002.

4. GenBank AF287270, 2000.

5. Declaration of Weiman Sun, Ph.D. Under 37 CFR § 1.132.

Appendix C: Related Proceedings Appendix

None.

Research Report

Design Strategies and Performance of Custom DNA Sequencing Primers

BioTechniques 27:528-536 (September 1999)

G.A. Buck[1], J.W. Fox[2], M. Gunthorpe[3], K.M. Hager[4], C.W. Naeve[5], R.T. Pon[6], P.S. Adams[7] and J. Rush[8]
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ABSTRACT

This study surveyed strategies of sequencing primer selection and evaluated primer performance in automated DNA sequencing. We asked participants to relate their preferred primer design strategies to identify primer characteristics that are considered most important in sequencing primer design. The participants preferred primers of 18–24 nucleotides (nt), 39%–58% G+C, a melting temperature (T_m) of 53°–65°C with a 1–2 nt 3' GC clamp, hairpin stems of less than 2–3 bp, homopolymeric runs of less than 4–5 nt, primer dimers of less than 3–4 bp and secondary priming sites of less than 3–4 bp. We provided a 300-bp test sequence and asked participants to submit sequences of 1–3 optimal sequencing primers. Submitted primers ranged from 17–24 nt and largely conformed to the preferred parameters. Submitted primers were distributed across the test sequence, although some sites were disfavored. Surprisingly, approximately 45% of the primers were selected "manually", more than by any software package. Each of 69 submitted and 95 control primers, distributed at 3-bp intervals across the test sequence, were synthesized, purified and tested using a Model 377 PRISM™ DNA Sequencer with dichlororhodamine dye terminator reagents (dRhodamine dye terminators). Approximately half of the control primers were also tested using rhodamine dye terminator reagents ("old" rhodamine dye terminators). The results indicated that primer physico-chemical characteristics thought to have a strong impact on sequencing performance had surprisingly little effect. Thus, primers with high or low percent G+C or T_m, strong secondary priming scores or long 3' homopolymeric stretches yielded excellent sequences with the dRhodamine dye terminator reagents, although these characteristics had a stronger effect when the old rhodamine reagents were used. The old rhodamine reagents gave sequences with a similar average read length, but the number of errors and ambiguities or "N's" was consistently higher. Moreover, the effects of the primer physico-chemical characteristics were also more evident with the old rhodamine dyes. We conclude that under optimal sequencing conditions with highly pure template and primer, many of the commonly applied primer design parameters are dispensable, particularly when using one of the new generation of sequencing reagents such as the dichlororhodamine dye terminators.

INTRODUCTION

The Nucleic Acids Research Committee (NARC) of the Association of Biomolecular Resource Facilities (ABRF) evaluates the procedures and performance of research facilities that perform nucleic acid synthesis and sequencing in academic, industrial and other institutions (5,6). These studies provide a measure of the industry state-of-the-art, as well as the level of performance, that can be expected from such facilities. Previous projects have examined general operations of DNA core laboratories (15), the accuracy of automated DNA sequencing in these facilities (12), and the performance of unpurified synthetic oligonucleotides as primers for automated DNA sequence analysis (14). The DNA Sequencing Research Committee of the ABRF has performed studies evaluating the ability of DNA core facilities to sequence difficult templates (2) and the performance of various sequencing strategies and chemistries (1). Herein, we have studied primer selection strategies used by DNA sequencing facilities and empirically examined the quality of the sequence data provided by primers selected by these facilities.

Despite a long-standing conviction within the DNA sequencing community that careful primer design is essential to ensure high quality data (c.f., 11,14,17,18), there is a paucity of empirical studies supporting this view. A variety of reasonable "rules of thumb" (13) suggesting optimal ranges for primer length, percent G+C, melting

Table 1. Predicted Optimal and Observed Physico-Chemical Characteristics of Primers for Automated DNA Sequencing or PCR

	Sequencing Primers[a]		PCR Primers[b]		Submitted Primers[c]	
	Optimum[d]	Range[e]	Optimum[d]	Range[e]	Mean[f]	Range[g]
Length	18–24 nt	15–40 nt	18–29 nt	15–40 nt	19.7 nt	16–24 nt
T_m[h]	52°–66°C	40°–95°C	50°–69°C	40°–95°C	66°C	48°–91°C
Percent G+C of primer	40%–61%	30%–70%	40%–61%	30%–70%	53%	29%–82%
3′ GC clamp[i]	1–2 nt	0–5 nt	1–2 nt	0–2 nt	1.1 nt	0–4 nt
Primer dimer[j]	3–4 bp	0–7 bp	3–4 bp	0–7 bp	-5.4	-1.6–16.5
Hairpin stems[k]	2–3 bp	0–6 bp	2–3 bp	0–6 bp	1.1 bp	0–5 bp
Homopolymer runs[l]	4–5 nt	3–10 nt	4–5 nt	3–10 nt	3.0 nt	2–5 nt
Secondary priming[m]	3–4	0–10 bp	3–4 bp	0–10 bp	49[n]	0–266[n]

[a]Parameter values preferred by participants for primers to be used in sequencing.
[b]Parameter values preferred for primers to be used in PCR experiments.
[c]Parameter values of the primers submitted by participants.
[d]Optimum values for each parameter as selected by the participants.
[e]The range of acceptable values selected by the participants.
[f]The mean values for each parameter calculated from the submitted primers.
[g]The range of values for each parameter calculated from the submitted primers.
[h]Values submitted by participants for primers to be used for sequencing or PCR and nearest neighbor T_m of primers submitted by participants.
[i]The 3′ clamp refers to the number of G or C bases at the 3′ terminus of the primer.
[j]Largest number of contiguous complementary bases permitting dimerization of primer or the stability of strongest potential primer dimer (kcal) as measured by Oligo 5.0.
[k]Largest number of bases capable of forming a hairpin stem in the primer.
[l]Number of bases in longest homolopolymeric run in the primer.
[m]Number of bases permitted in most stable secondary priming site.
[n]The priming efficiency as calculated by Oligo 5.0 for test sequence only (submitted primers).

temperature (T_m) and composition have led to the development of primer design software packages that identify primers conforming to these criteria (4,16,18). However, the success of these strategies in selection of high-quality sequencing primers, to our knowledge, has not been extensively examined. In many sequencing projects, primer design and synthesis represent the most significant costs and consume the bulk of effort and time. Therefore, it is extremely important that these primers yield high-quality sequence data.

To examine this issue, an e-mail survey containing general questions about laboratory functions and specific questions concerning important characteristics for primer selection was distributed. A 300-bp test sequence was provided for which participants were asked to design sequencing primers. The submitted primers and a set of control primers spanning the 300-bp test sequence at 3-bp intervals were synthesized and used to sequence the test template on a Model 377 PRISM™ Automated DNA Sequencer (PE Biosystems, Foster City, CA, USA). Due to the results of this study, current primer design rules of thumb may be streamlined to facilitate more efficient primer selection for sequencing projects.

MATERIALS AND METHODS

The Survey

In December of 1996 and January of 1997, members of the ABRF were requested via e-mail distributions, the ABRF Electronic Bulletin Board and a standard mailing, to participate in a study of DNA sequencing primer design. The distribution provided a 300-bp "test sequence" (Figure 1) that was selected and previously shown to contain no segments that affect sequence ladder extension (data not shown). Participants were asked to use the prevailing technology in their laboratories to design and submit the sequences of up to three sequencing primers in the forward direction. Participants were requested to select the best primers irrespective of their positions on the test sequence. All responses were anonymously screened and coded by Virginia Commonwealth University (VCU) Health Sciences Computer Center personnel. Identifying data were removed from each document before being forwarded to the Committee for analysis. Several respondees submitted uninterpretable data. Where possible, these respondees were identi-

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fied by Computer Center personnel, and new information was requested. Every effort was made to maintain the integrity of the data and the anonymity of the participants.

Control and Submitted Primers

A panel of 95 primers were synthesized as controls. These 18 nucleotide (nt) primers spanned the 300-bp test sequence with their 5′ termini located at 3-bp intervals (i.e., the first began at bp 1 of the test sequence, the second began at bp 4, etc.). All primers were analyzed with the Oligo™ Program 5.0 (NBI/Genovus, Plymouth, MN, USA) (16) and the number of bases, percent G+C, T_m (nearest neighbor method described in Reference 9), internal structure, secondary priming sites, etc., were recorded.

The 69 submitted and 95 control primers were synthesized on a Model 3948 DNA Synthesis and Purification System (PE Biosystems) at three different sites. Synthesis was performed at the 40 nmol scale using PE Biosystems reagents and the standard synthesis, cleavage and purification cycles. An aliquot of each sample was analyzed by polyacrylamide gel electrophoresis (PAGE) and by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF/MS). PAGE was performed using precast minigels (Novex, San Diego, CA, USA) following the protocol specified by the manufacturer. Mass analysis was performed on a 200-pmol sample mixed with 10 mg/mL 3-hydroxypicolinic acid matrix (in 50% acetonitrile, 0.1% trifluoracetic acid) on a Voyager RP MALDI-TOF/MS instrument (PerSeptive Biosystems, Framingham, MA, USA). Criteria for passing quality control were: (*i*) a single major band on the gel; (*ii*) appropriate mobility on the gel compared to molecular weight standards; (*iii*) a synthesis yield >0.5 ODU (absorption of sample in 1 mL at 260 nm with 1 cm pathlength set up on the 3948 Synthesizer); and (*iv*) a single peak of the expected mass by MALDI-TOF/MS. Any oligonucleotides that failed to meet these criteria were resynthesized.

Test Template and Preparation

The plasmid template was preselected to contain a test sequence lacking obstacles to sequence extension (data not shown) and purified by double banding in CsCl-ethidium bromide isopycnic density gradients (10).

DNA Sequence Analysis

Each of the purified and quality-tested oligonucleotides were used as a primer for DNA sequence analysis using dRhodamine Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® FS reagents (PE Biosystems) under standard reaction conditions as described by the manufacturer at 20 µL total vol containing 300 ng of template and 5 pmol of primer. Sequencing reactions were run for 25 cycles of 10 s at 96°C, 5 s at 50°C and 4 min at 60°C in a GeneAmp® PCR System 9700 DNA Thermal Cycler (PE Biosystems). Reactions were analyzed on Model 377 PRISM Automated DNA Sequencers using 5% Long Ranger™ (FMC BioProducts, Rockland, ME, USA) gels with Tris-Borate-EDTA buffer (TBE; pH 8.3). Approximately 30% of the control primers were rerun using the same dRhodamine dye terminator/Taq FS kit to ensure reproducibility, and approximately 50% of the control primers were rerun using the old rhodamine dye terminator/Taq FS kit (PE Biosystems). The dRhodamine or dichlororhodamine reagents are modifications of the original old rhodamine dyes that have narrower emission spectra, less spectral overlap and more even peak heights than the earlier dyes (19). Each 5% polyacrylamide sequencing gel was electrophoresed for approximately 8 h, and the data was directly exported for analysis using Sequencher™ (Gene Codes, Ann Arbor, MI, USA). Using this software, the raw data was trimmed to remove 5′ and 3′ ambiguous sequences, so that the first and last 25 nt contained no ambiguities. Each result was aligned with the known sequence, and we recorded: (*i*) the number of nt between the 3′ end of the primer and the beginning of the trimmed data; (*ii*) the number of nt in each sequence read; and (*iii*) the number of errors, including miscalls, insertions, deletions and ambiguities in each 100-bp window through the end of the sequence read.

```
  1  ATCCACACGG CGCTTTGACT CCCCTTCTGA AAAAAAAGAA CACGTTGATT
 51  CCCCTTTTGA TTTCCCACGA ATGCGAAGCA CAATTCACGC ACAGCCAACG
101  GCAGGGAGAG ACGATAAGGT AAAATACACA CAAGTGGTGG AATCACAAAA
151  GGACACGCAC AGCACGCAAA TAAACATTGC ACACGGCATG ACATTTTGCG
201  ACCCACACAC AATGTGTACA CTACAATTAT ATGAAAATCC CTCCCCCATT
251  CCGCGGGTGC GCCGCAAAGG GCCAAAAACC CAAATGATCC ACTTTATTAT
```

Figure 1. Test sequence. Shown is the 300-bp sequence distributed to participants for design of forward sequencing primers. Participants were asked to design primers in the forward direction.

RESULTS

Participant Profiles

Thirty-nine laboratories participated in the study, submitting 69 primer sequences. Of the participants, approximately 77% provide synthesis services and approximately 85% provide sequencing services, but only approximately 50% provide primer design or walking services, and less than one third offer template preparation. Laboratories performing these services averaged annually approximately 3700 syntheses, 7000 sequence runs, 425 primer designs, 424 template purifications and less than 200 primer walking projects. Charges for services averaged \$1.17/nt for 40–50 nmol and \$2.19/nt for 150–200 nmol syntheses of a 25-nt unpurified primer and \$24/reaction for DNA sequence analysis (\$9 for gel only). The average price for a primer walking sequencing project of a 2-kbp insert in a plasmid vector was approximately \$500 or \$0.25/bp. Charges for "external users", where available, averaged approximately 17%–50% higher than charges for institutional users. DNA synthesis and sequencing instrumentation was dominated by PE Biosystems; i.e., 77% of synthesizers and 92% of sequencers, but Beckman

Instruments, PerSeptive Biosystems and LI-COR were also represented. Only approximately 15% of the participants maintained robotic systems for template preparation or sequencing. Finally, when asked if commercial suppliers had an impact on services provided by the facilities, almost 80% of the laboratories responded affirmatively. Approximately 38% had dropped

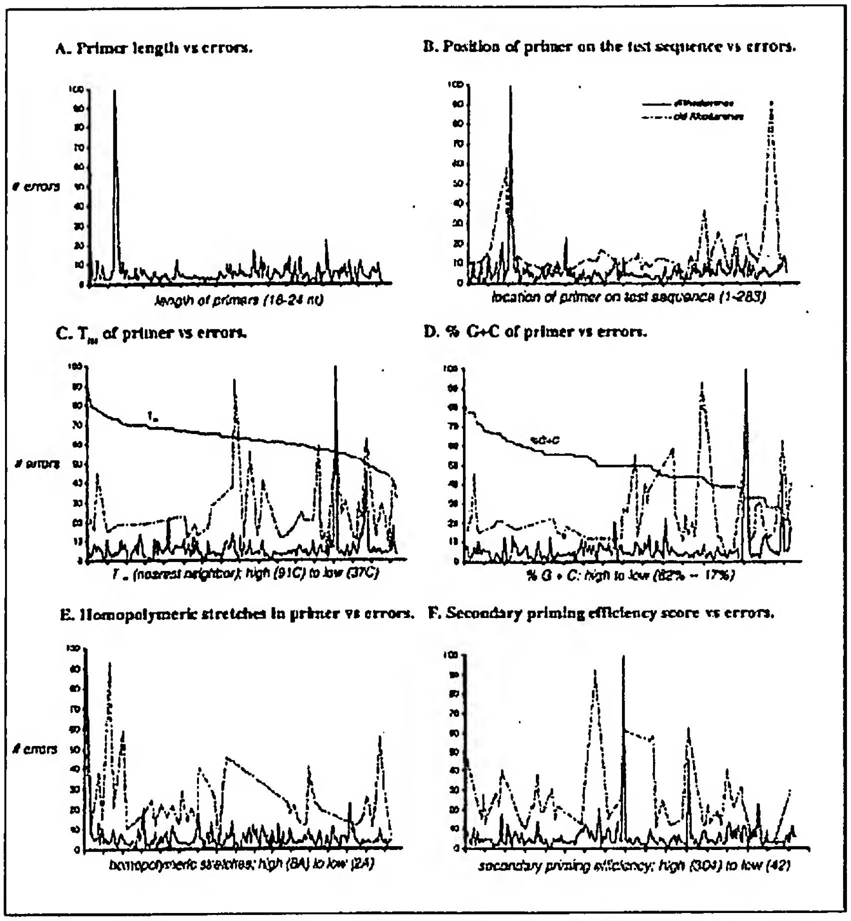

Figure 2. Primer characteristics vs. sequencing performance. The performance of sequencing primers, sorted according to various physico-chemical characteristics, was examined. The number of errors over the full-length read of each primer, determined as described in the Materials and Methods, was plotted against the results of each primer sorted according to the relevant parameter. (A) Primer length vs. errors. The primers were sorted according to their lengths in ascending order, and secondarily, according to their positions on the test sequence. The number of errors over the full-length read is plotted. Note that since all of the primers tested with the old rhodamine reagents were controls, and therefore 18-nt long, the data for old rhodamines are not included in this analysis. The X-axis distributes the primers from 16–24 nt in length; the y-axis indicates the number of errors over the full-length read of that primer. (B) Position of the primer on the test sequence. The primers were sorted according to the position of their 5′ nt on the test sequence (bp 1–283 on x-axis) and the number of errors in their full-length reads were plotted (y-axis). (C) T_m of the primer. The primers were sorted in descending order by their T_m's (91°–37°C on x-axis) and the number of errors in their full-length reads were plotted (y-axis). (D) The percent G+C of the primer. The primers were sorted in descending order (82%–17% on x-axis) by their percent G+C, and the number of errors in their full-length reads were plotted (y-axis). (E) Homopolymeric stretches in the primer. The primers were sorted according to first the number of nt (8–2 on x-axis) in the longest homopolymeric stretch, and secondarily according to the number of nt in the second longest homopolymeric stretch. The number of errors over the full-length read were plotted (y-axis). (F) Secondary priming efficiency of the primers. Each primer was examined for secondary priming sites in the vector and across the test sequence using Oligo 5.0. The priming efficiency score assigned by Oligo 5.0 was determined. The primers were sorted first according to the score of the strongest secondary priming site in the vector (304– 42 on x-axis) and secondarily according to the strongest secondary priming score in the test sequence. The number of errors across the full-length reads were plotted (y-axis).

ppm), due to the β effect. The same effect for compound **4** shifts the C-6 signal, which is observed at 66.2 ppm. Deprotection of HO-6 also affects the pattern of H-6,6′ in the ^{1}H NMR spectrum of **3**. In this case, the two protons appear as a doublet at the same chemical shift (4.07 ppm, Table 1).

Oxidation of HO-6 was performed with PCC as described (12) affording **5** (88%). Compound **5** showed a singlet at 9.2 ppm in the ^{1}H NMR spectrum, and a signal at δ 196.5 in the ^{13}C NMR spectrum, both diagnostic signals for the presence of the aldehyde group at C-6. For the reduction and debenzoylation steps, standard procedures were used. Compound [^{3}H]**1** showed the same chromatographic properties by TLC (Fig. 1, lane I) and HPAEC-PAD (Fig. 2A) than the nonradioactive compound. The specific activity (13 μCi/μmol) was calculated from the ratio between the radioactivity of the compound eluted from the HPAEC and the amount of material calculated by using mannose as standard.

Compound [^{3}H]**1** was used as substrate for the *exo*-β-D-galactofuranosidase from *P. fellutanum*, under the usual conditions (6). The enzymatic reaction could be followed by TLC (Fig. 1, lane II) and HPAEC-PAD analysis (Fig. 2B).

This is the first report on the synthesis of a radioactive substrate for β-D-galactofuranosidase. The radiolabeled material will facilitate detection of the enzyme in cultures or cellular fractions of microorganisms. In addition to being more sensitive, it can be used with colored biological materials without the interference caused in the colorimetric assay. The method could be useful for labeling other substrates in studies on the biosynthesis of galactofuranose glycans, like the galactan of *Mycobacterium tuberculosis* (13), although this chemical-labeling procedure would not be specific when other monosaccharides are present.

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Rapid Genotype Method to Distinguish Frequent and/or Functional Polymorphisms in Human *N*-Acetyltransferase-1

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Human *N*-acetyltransferase 1 (NAT1)[2] catalyzes the *N*-acetylation of arylamine and hydrazine drugs and the O-acetylation of N-hydroxylated metabolites of ar-

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[2] Abbreviations used: NAT1, *N*-acetyltransferase 1; NAT2, *N*-acetyltransferase 2; PCR, polymerase chain reaction; FAM, 6-carboxyfluorescein; TET, tetrachloro-6-carboxyfluorescein; MGB, minor groove binder; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism; UTR, untranslated region.

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TABLE 1

Frequency of *NAT1* Alleles in American and French Populations Determined by Comprehensive RFLP and SSCP Genotyping Methods

NAT1 allele	Nucleotide substitutions[a]	Number (%) identified in control populations			
		USA (Iowa)[b]	France[c]	Canada[d]	Germany[e]
*NAT1*4*	None (reference)	487 (74.2)	402 (74.4)	135 (70.3)	497 (72.4)
*NAT1*3*	**C^{1095}A**	20 (3.0)	15 (2.8)	0	21 (3.1)
*NAT1*10*	**T^{1088}A, C^{1095}A**	114 (17.4)	96 (17.8)	48 (25.0)	140 (20.4)
*NAT1*11* (*A* or *B*)	C^{-344}T, A^{-40}T, **G^{445}A**, G^{459}A, T^{640}G, 9-bp deletion 1065–1090, **C^{1095}A**	8 (1.2)	0	4 (2.1)	20 (2.9)
*NAT1*14* (*A* or *B*)	**G^{560}A, T^{1088}A, C^{1095}A**	13 (2.0)	20 (3.7)	5 (2.6)	4 (0.6)
*NAT1*15*	**C^{559}T**	2 (0.3)	1 (0.2)	0	0
*NAT1*17*	**C^{190}T**	7 (1.1)	3 (0.5)	0	1 (0.1)
*NAT1*19*	**C^{97}T**	0	0	0	0
*NAT1*22*	**A^{752}T**	5 (0.8)	0	0	3 (0.4)
*NAT1*26B*	TAA^{1091} insertion	0	1 (0.2)	0	0
*NAT1*28*	TAATAA$^{1085-90}$ deletion	0	1 (0.2)	0	0
*NAT1*29*	T^{1025} deletion, **T^{1088}A, C^{1095}A**	0	1 (0.2)	0	0
Total NAT1 alleles		456	540	192	686

[a] Nucleotide substitutions (compared with *NAT1*4* reference) detected in the new genotype method are in boldface.
[b] Reference (13).
[c] Reference (16).
[d] Reference (14).
[e] Reference (15).

TABLE 2

Primers and Fluorogenic Probes for SNP Determination[a]

97-Forward primer (58–85)	5′-gacttggaaacattaactgacattcttc-3′
97-Reverse primer (131–107) [74 bp]	5′-caatggatgttaaggttctcaaagg-3′
97C-TaqMan MGB probe (90–104)	FAM-ccagatc**C**gagctgt
97T-TaqMan MGB probe (90–105)	VIC-ccagatc**T**gagctgtt
190-Forward primer (143–165)	5′-tggacttaggcttagaggccatt-3
190-Reverse primer (220–198) [78 bp]	5′-gatgattgacctggagacaccat-3′
190C-TaqMan MGB probe (196–185)	FAM-caccccGatttc
190T-TaqMan MGB probe (195–183)	VIC-accccAatttctt
445-Forward primer (395–416)	5′-ggcagcctctggagttaatttc-3′
445-Reverse primer (506–481) [70 bp]	5′-tactgttcccttctgatttggtctag-3′
445G-TaqMan MGB probe (439–452)	FAM-ccttgt**G**tcttccg
445A-TaqMan MGB probe (437–454)	VIC-tgccttgt**A**tcttccgtt
559/560-Forward primer (497–521)	5′-gggaacagtacattccaaatgaaga-3′
559/560-Reverse primer (595–571) [99 bp]	5′-ttgttcgaggcttaagagtaaagga-3′
559C/560G-TaqMan MGB probe (552–566)	FAM-caaatac**CG**aaaaat
559C/560A-TaqMan MGB probe (552–569)	VIC-caaatac**CA**aaaaatcta
559T/560G-TaqMan MGB probe (552–567)	TET-caaatac**TG**aaaaatc
752-Forward primer (713–734)	5′-ccctcacccataggagattcaa-3′
752-Reverse primer (793–765) [81 bp]	5′-tttctatttcttcctcactcagagtcttg-3′
752A-TaqMan MGB probe (743–762)	FAM-acaatacag**A**tctaatagag
752T-TaqMan MGB probe (743–762)	VIC-acaatacag**T**tctaatagag
1088/1095-Forward primer (1041–1066)	5′-gaaacataaccacaaaccttttcaaa-3′
1088/1095-Reverse primer (1136–1112) [96 bp]	5′-aaatcaccaatttccaagataacca-3′
1088T/1095C-TaqMan MGB probe (1105–1079)	FAM-atctttaaaa**G**acattt**A**ttattatta
1088A/1095A-TaqMan MGB probe (1106–1079)	VIC-catctttaaaa**T**acattt**T**ttattatta
1088T/1095A-TaqMan MGB probe (1106–1078)	TET-catctttaaaa**T**acattt**A**ttattattat

[a] Nucleotide positions are indicated in parentheses. PCR product size is indicated in square brackets.

omatic and heterocyclic amines (1–10). NAT1 is encoded by a single 870-bp open reading frame that exhibits genetic polymorphism in human populations (11). Numerous studies have shown that the *NAT1* genetic polymorphism is associated with predisposition to various cancers from arylamine and heterocyclic amine carcinogens (reviewed in 12). The frequency of *NAT1* alleles has been difficult to assess because, until recently, most *NAT1* genotype data in the literature were based on the original method designed to detect only four *NAT1* alleles (12). More recently, comprehensive analyses of *NAT1* polymorphisms have been conducted by restriction fragment length polymorphism (RFLP) analysis (13–15) and single-strand conformation polymorphism (SSCP) analysis (16). As shown in Table 1, the comprehensive RFLP genotype methods identified eight *NAT1* alleles in American, Canadian, and German populations and the comprehensive SSCP genotype method identified nine *NAT1* alleles in a French population. However, three of these *NAT1* alleles (*NAT1*26B, *28,* and **29*) were identified in only one French subject and were not identified in the other ethnic groups. Consistent with these findings, our laboratory recently completed automated DNA sequencing of more than 1300 *NAT1* alleles from a Polish population and more than 1000 *NAT1* alleles from a Dutch population. The *NAT1* alleles identified following DNA sequencing were *NAT1*3, *4, *10, *11, *14, *15, *17,* and **22* (unpublished data). *NAT1* alleles with nucleotide substitutions in the coding region were recently expressed in yeast and those that possessed the $C^{97}T$, $C^{190}T$, $C^{559}T$, $G^{560}A$, or $A^{752}T$ nucleotide polymorphisms exhibited reduced *N*- and *O*-acetyltransferase activities (10). Relationships between *NAT1* genotype and phenotype in humans also have been reported (6, 9, 14, 17–21). The $T^{1088}A$ and $C^{1095}A$ polymorphisms in the 3′-untranslated region (UTR) are frequent (Table 1) and may confer functional consequences (18, 20, 21). The $G^{445}A$ polymorphism also may have functional consequences (4) and is necessary to distinguish the *NAT1*11A* and *B* alleles. Since frequencies of *NAT1* nucleotide polymorphisms have been determined by comprehensive RFLP, SSCP, and DNA sequencing methods, and since functional effects of the nucleotide polymorphisms have been assessed, we designed a rapid genotyping method to distinguish among the frequent and/or functional nucleotide polymorphisms in *NAT1* analogous to a genotype method recently designed for *NAT2* (22).

Nucleotide-specific polymerase chain reaction (PCR) primers and fluorogenic probes were designed using Primer Express (Version 1.5, Applied Biosystems, Foster City, CA) (Table 2). The fluorogenic MGB probes were labeled with a reporter dye (either FAM or VIC or TET) specific for one of the possible nucleotides identified at eight polymorphisms in the *NAT1* coding re-

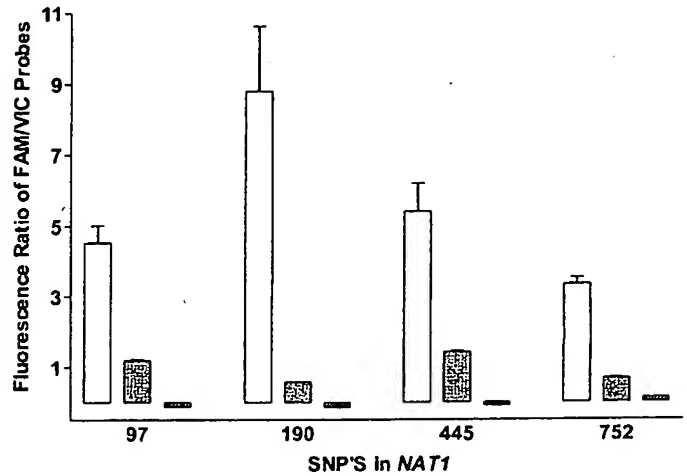

FIG. 1. Fluorescence ratio of FAM-labeled/VIC-labeled fluorogenic probes specific for the nucleotide polymorphisms in the *NAT1* coding region. Each bar represents the mean ± SE for determinations in DNA from three human subjects. Open bars represent DNA samples homozygous for 97C, 190C, 445G, and 752A, respectively. Solid bars represent DNA samples homozygous for 97T, 190T, 445A, and 752T, respectively. Crossed bars represent DNA samples heterozygous for each nucleotide polymorphism. The fluorescence ratio differed significantly ($P < 0.005$) among homozygous and heterozygous *NAT1* genotypes for each nucleotide polymorphism following one-way analysis of variance.

gion or 3′-UTR: $C^{97}T$ (R^{33}Stop), $C^{190}T$ ($R^{64}W$), $G^{445}A$ ($V^{149}I$), $C^{559}T$ (R^{187}Stop), $G^{560}A$ ($R^{187}Q$), $A^{752}T$ ($D^{251}V$), $T^{1088}A$ (3′UTR), $C^{1095}A$ (3′UTR). The fluorogenic probes for the 559/560 and the 1088/1095 are distinguished using a three-probe system that has both nucleotide polymorphisms in the same probe. This was necessary because the 559/560 and 1088/1095 are too close together to use the conventional two-probe TaqMan assay. The nucleotide-specific primers amplify a segment of the *NAT1* gene flanking the probes.

TaqMan Universal PCR Master Mix (Applied Biosystems) was used to prepare the PCR. The 2× mixture is optimized for TaqMan reactions and contains AmpliTaq Gold DNA polymerase, AmpErase, dNTPs with UTP, and a passive reference. Primers, probes, and DNA were added to final concentrations of 300 nM, 100 nM, and 0.5–2.5 ng/μl, respectively. Controls (no DNA template) were run to ensure that there was no amplification of contaminating DNA. The amplification reactions were carried out in an ABI Prism 7700 sequence detection system (Applied Biosystems) with two initial hold steps (50°C for 2 min, followed by 95°C for 10 min) and 40 cycles of a two-step PCR (92°C for 15 s, 60°C for 1 min). The fluorescence intensity of each sample was measured at each temperature change to monitor amplification of the *NAT1* gene. The nucleotide present in the *NAT1* alleles was determined by the fluorescence ratio of the nucleotide-specific fluorogenic probes. The fluorescence signal increases when the

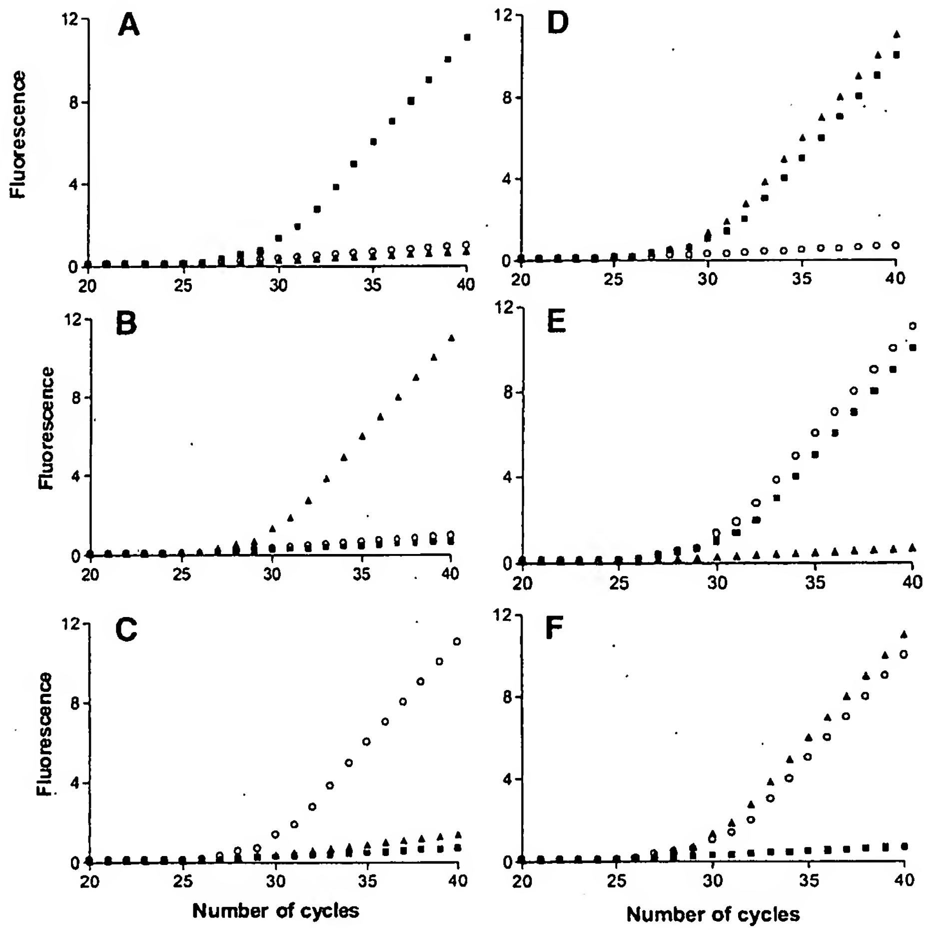

FIG. 2. Illustration of three-probe assay designed to distinguish 1088/1095 or 559/560 nucleotide combinations. The fluorescence signal is plotted as a function of cycle number. In each panel, squares represent signal from FAM-labeled probes, triangles represent signal from VIC-labeled probes, and circles represent signal from TET-labeled probes. (A) 1088TT/1095CC or 559CC/560GG; (B) 1088AA/1095AA or 559CC/560AA; (C) 1088TT/1095AA or 559TT/560GG; (D) 1088TA/1095CA or 559CC/560GA; (E) 1088TT/1095CA or 559CT/560GG; (F) 1088TA/1095AA or 559CT/560GA.

probe with the exact sequence match binds to the single-stranded template DNA and is digested by the 5′–3′-exonuclease activity of AmpliTaq Gold DNA polymerase (Applied Biosystems). Digestion of the probe releases the fluorescent reporter dye (either FAM or VIC or TET) from the quencher.

As shown in Figs. 1 and 2, the method readily distinguishes between the two possible nucleotides at each of the eight nucleotide polymorphisms identified in the *NAT1* coding and 3′-UTR: $C^{97}T$ (R^{33}Stop), $C^{190}T$ ($R^{64}W$), $G^{445}A$ ($V^{149}I$), $C^{559}T$ (R^{187}Stop), $G^{560}A$ ($R^{187}Q$), $A^{752}T$ ($D^{251}V$), $T^{1088}A$ (3′-UTR), $C^{1095}A$ (3′-UTR). Determination of the eight nucleotide polymorphisms showed complete concordance with a comprehensive RFLP method (13) and DNA sequencing. All *NAT1* alleles (*NAT1*3, NAT1*4, NAT1*10, NAT1*11A, NAT1*11B, NAT1*14A, NAT1*14B, NAT1*15, NAT1*17, NAT1*19, NAT1*22*) except for very rare ones can be detected with this method. Major advantages of this genotyping method are that it does not require post-PCR processing or the use of radioactivity. Since the method amplifies a much smaller segment of *NAT1* (74–99 bp) than is required for the comprehensive RFLP (1148 bp) or SSCP (1197 bp) genotyping methods (13, 16), it is much more effective in determining *NAT1* genotypes in human DNA samples derived from buccal cells or paraffin-embedded tissues. Since the new method does not require post-PCR processing, it is much faster and suitable for automated, high-throughput applications.

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Report

Carrier Screening for Mucolipidosis Type IV in the American Ashkenazi Jewish Population

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Mutations in the *MCOLN1* gene cause mucolipidosis type IV (MLIV), a severely debilitating, autosomal recessive, lysosomal storage disorder. Approximately 80% of patients with MLIV are of Ashkenazi Jewish (AJ) descent, and two mutations, IVS3−2A→G and 511del6434, account for >95% of the mutant alleles in this population. To determine the carrier frequencies of these two mutations, 2,029 anonymous, unrelated, unaffected AJ individuals from the greater New York metropolitan area were screened. A multiplex PCR method coupled with allele-specific oligonucleotide hybridization was developed, to enable large-scale screening. The frequencies of the IVS3−2A→G and 511del6434 mutations were 0.54% and 0.25%, respectively, for a combined carrier frequency of 0.79%, or 1 in 127 individuals (95% CI 0.40%–1.17%). The addition of both AJ mutations causing this neurodegenerative disorder should be considered for prenatal carrier screening in this population.

Mucolipidosis type IV (MLIV [MIM 252650]) is a neurodegenerative lysosomal storage disorder that is inherited as an autosomal recessive trait. Clinically, the disease, which occurs primarily among Ashkenazi Jewish (AJ) individuals, is characterized by growth and psychomotor retardation, as well as ophthalmologic abnormalities, which include corneal clouding, progressive retinal degeneration, and strabismus (Berman et al. 1974; Riedel et al. 1985). There is clinical variability, but most patients never develop the ability to speak or walk and remain at a developmental level of age 1–2 years. Although the disorder is associated with the accumulation of cytoplasmic storage bodies, normal levels of lysosomal hydrolases are present, with no specific identifiable storage compound (Amir et al. 1987). Electron photomicrographs of tissue biopsies from patients with MLIV have shown a widespread heterogeneous accretion of lipids and water-soluble compounds, indicating that the etiology of the disease is probably not related to the deficiency of a particular lysosomal enzyme but rather to a defect in endocytosis (Bargal and Bach 1997; Chen et al. 1998). It has been suggested that many cases may not be properly diagnosed, because of the variability in the observed phenotype and the lack of an indicative storage compound.

The MLIV locus was mapped to chromosome 19p13 in AJ families, and haplotype analysis of these families indicated that there were two founder chromosomes that accounted for 73% (major haplotype) and 23% (minor haplotype)—a total of 96%—of the carrier chromosomes in the AJ population (Slaugenhaupt et al. 1999; Sun et al. 2000). Recently, the gene that is mutated in patients with MLIV, *MCOLN1* (MIM 605248), was isolated, and the two AJ founder mutations were identified. These mutations were (1) a splicing mutation, IVS3−2A→G, which resulted in skipping of exon 4, with the subsequent loss of the reading frame, and (2) a 6,434-bp deletion spanning genomic nucleotides 511–6,944, designated "511del6434," which included the 5′ flanking region of the gene through exon 7 (Bargal et al. 2000; Bassi et al. 2000; Sun et al. 2000). Since both mutations resulted in the loss of a functional gene product, the severity of the phenotype was similar in patients who were homoallelic or heteroallelic for these mutations.

The *MCOLN1* gene encodes the putative protein mucolipin-1, which is 580 amino acids long and is somewhat homologous to the polycystins, a family of cation channels (Somlo and Ehrlich 2001). In *Caenorhibditis*

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elegans, studies of cup-5, the mucolipin-1 homolog, have indicated that these proteins are vesicle membrane-associated regulators of the endocytic pathway, where loss of cup-5 function results in large lysosomal or late endosomal structures that exhibit both an increase in the rate of uptake of fluid phase markers and a decrease in the ability to degrade proteins (Fares and Greenwald 2001). The function of mucolipin-1 that is predicted on the basis of these studies is consistent with the lysosomal pathology observed in cultured cells derived from patients with MLIV.

To determine the frequency of carriers for MLIV in the AJ population, we screened for both the major IVS3−2A→G and the minor 511del6434 mutations in genomic DNAs isolated from blood samples obtained from 2,029 unrelated AJ individuals from the New York metropolitan area who were referred for prenatal carrier testing for other genetic disorders prevalent in the AJ population. DNA was extracted from peripheral blood obtained by venipuncture and archived with informed consent for use in research studies. All personal identifiers were removed, and the samples were tested anonymously. A multiplex PCR amplification was performed for 28 cycles with 100 ng of genomic DNA in a volume of 50 μl containing 10 pmol of each primer tagged with a 5′ universal primer sequence (UPS) (Shuber et al. 1995). The primer sequences designed to detect the major mutation were MLIV-1UPS (5′-GCGGTCCCAAAAGGGTCAGTATCTTCCATGCTGTGGACCA-3′) and MLIV-2UPS (5′-GCGGTCCCAAAAGGGTCAGTAACAGTGAAGCCTCGTCCTG-3′), and the primer sequences designed to detect the minor mutation were MLIV-3UPS (5′-GCGGTCCCAAAAGGGTCAGTGGCAGCTTTCTCAATGAAGG-3′ and MLIV-4UPS (5′-GCGGTCCCAAAAGGGTCAGTTCACCGTGCTGGAAGACACT-3′). In addition, 100 mM of each dNTP (Roche Molecular Biochemicals), 5 U of *Taq* DNA polymerase (Roche), 10 mM Tris-HCl, 50 mM KCl, 0.1% TritonX-100, and 1.5 mM $MgCl_2$ were added to the PCRs. Dimethyl sulfoxide (Sigma) was added to a final concentration of 4%, because of the high (65%) GC content of the region amplified for analysis of the major mutation. The primers were designed from the genomic sequence of the *MCOLN1* gene (Sun et al. 2000; GenBank accession number AF287270) to amplify a 410-bp PCR product encompassing the IVS3−2A→G mutation and a 396-bp PCR product when a 511del6434 mutation was present (fig. 1). Therefore, the multiplex PCR amplification of DNA from individuals with neither mutation and individuals who were heterozygous or homozygous for the IVS3−2A→G mutation would result in a single PCR product of 410 bp (fig. 1). Amplification of DNA from individuals who were carriers of the 511del6434 mutation or heteroallelic for both mutations would result in PCR products of 396 bp and 410 bp, respectively, whereas amplification of DNA from individuals who were homozygous for the 511del6434 mutation would result only in products of 396 bp (fig. 1). Control DNAs were obtained from the fibroblast cell lines GM02527, GM02529, and GM02525 (Coriell NIGMS Human Genetic Cell Repository), which were derived from patients with MLIV who were homozygous for the IVS3−2A→G mutation, compound heterozygous for the IVS3−2A→G/511del6434 mutations, and homozygous for the 511del6434 mutation, respectively (Bassi et al. 2000).

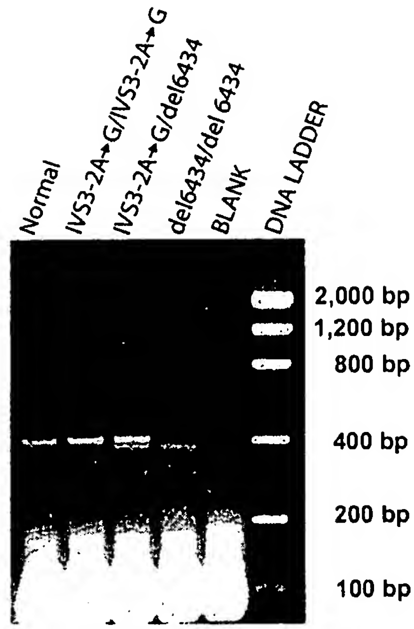

Figure 1 Multiplex PCR of the *MCOLN1* gene; 2% agarose gel showing the products that result from the multiplex PCR amplification of the *MCOLN1* gene with the IVS3−2A→G and 511del6434 primer pairs. Note that the 511del6434 primers amplify a product only when the deletion is present.

Aliquots (4 μl) of the PCR products were blotted in triplicate onto 8 × 12 cm Hybond −N+ membranes (Amersham Pharmacia Biotech) through use of a Biomek 2000 automated pipetting workstation (Beckman Coulter). Hybridization was performed with the following allele-specific oligonucleotides (ASOs): for the IVS3−2A→G mutation, 5′-TCTCTGCCCACAGTACCTG-3′ (normal) and 5′-TCTCTGCCCACGGTACCTG-3′ (mutant); and for the 511del6434 mutation, 5′-CCTGGGCTCAACAAAGCAC-3′. For radioactive detection, the membranes were hybridized, for a length of time ranging from 2 h to overnight, with ~10^6 counts per minute of γ-[^{32}P]ATP end-labeled probe/ml and 10-fold molar excess of the mutant or wild-type competitor oligonucleotide for the IVS3−2A→G mutation. The membranes were then washed sequentially in 5 × saline sodium citrate (SSC) for

5 min at room temperature and in 5 × SSC for 5 min at 45°C, followed by a wash in 0.1 × SSC/0.1% SDS for 5 min at 45°C. Filters were then exposed to autoradiography by use of Biomax MR film (Kodak) (fig. 2).

For the major IVS3−2A→G mutation, 2,029 AJ individuals were screened and 11 carriers were identified, corresponding to a frequency of 0.54%, or 1/184. Five carriers for the minor 511del6434 mutation were identified in a total of 2,029 AJ individuals tested, corresponding to a frequency of 0.25%, or 1/406. The combined frequency of the two mutations in the present study was, therefore, 0.79%, or 1/127 individuals (95% CI 0.40%–1.17%) (table 1). Because the haplotypes that correspond to these two mutations account for 96% of the carriers in the AJ population, the prevalences of the two mutations were 66% and 30% for the major and minor mutations, respectively. These percentages are in agreement with the results of Sun and colleagues (2000), who reported that the major and minor haplotypes are present on 73% and 23% of carrier chromosomes, respectively.

In a recent report (Wang et al. 2001), a small study of 123 AJ individuals was conducted, and a carrier frequency of 1/61 was reported for the major IVS3−2A→G mutation; however, no carriers for the minor 511del6434 mutation were identified (table 1). Therefore, the limited sample size prevented a reliable estimate of the frequency and distribution of MLIV carriers. Recently, Bargal et al. (2001) reported a carrier frequency of 1/100 for the two mutations in a sample of 2,000 AJ individuals from Israel (table 1). The distribution of the mutations was 94% for the major mutation and 6% for the minor mutation. This apparent disparity in the frequency of carriers between the AJ constituencies of the New York metropolitan area and Israel is not clear; however, the major mutation is more prevalent in the Israeli population and probably accounts for the observed higher frequency of total carriers of MLIV. These findings also may be indicative of different patterns of immigration of AJ individuals into the United States and Israel and/or may reflect distinct regional origins of the founders of the major and the minor mutation.

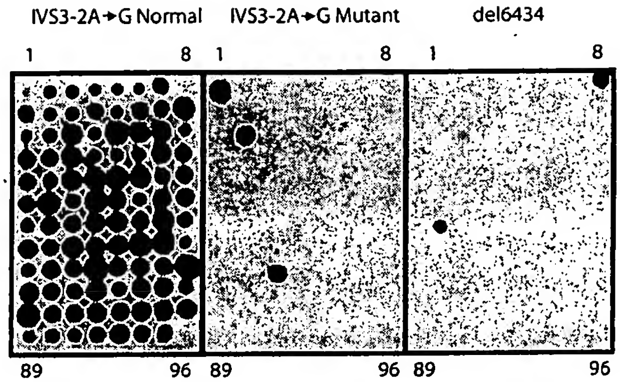

Figure 2 ASOs of the two AJ *MCOLN1* mutations. Shown are autoradiographs of dot blots hybridized with the normal IVS3−2A→G ASO (*left*), the mutant IVS3−2A→G ASO (*middle*), and the 511del6434 ASO (*right*). PCR samples 1–95 were amplified from genomic DNAs; sample 96 is the ddH_2O control. Sample 1 was amplified from the IVS3−2A→G homozygote (GM02527), sample 8 was amplified from the 511del6434 homozygote (GM02525), samples 18 and 67 were amplified from carriers of the IVS3−2A→G mutation, and sample 49 was amplified from a carrier of the 511del6434 mutation.

In a study of 17 Israeli AJ families with MLIV, familial origins were traced back to Poland or to neighboring Lithuania (Raas-Rothschild et al. 1999). The authors speculated that the underrepresentation of ultra-orthodox families among the 80 AJ families with MLIV worldwide might indicate a recent origin for the mutation, around the 18th and 19th centuries, in a founder that belonged to a secular family. These findings are likely to be specific for the major mutation, on the basis of its higher prevalence (94%) among the Israeli AJ population. Discordant carrier rates between Israeli and American Ashkenazi subpopulations have been reported recently for mutations in the *Connexin 26* gene, which cause nonsyndromic sensorineural recessive deafness

Table 1

Carrier Frequency of the *MCOLN1* Mutations Among Unrelated AJ Individuals

		IVS3−2A→G		511del6434		Combined	
Study (Population)	No. of Individuals	No. of Carriers	Carrier Percentage (Frequency)	No. of Carriers	Carrier Percentage (Frequency)	No. of Carriers	Carrier Percentage (Frequency)
Bargal et al. 2001 (Israel)	2,000	17	.85 (1/117)	1	.05 (1/2,000)	19[a]	.95 (1/106)[b]
Wang et al. 2001 (New York)	123	2	1.62 (1/62)	0	0	2	1.62 (1/62)
Present study (New York)	2,029	11	.54 (1/184)	5	.25 (1/406)	16	.79 (1/127)[c]
Combined	4,152	30	.72 (1/138)	6	.14 (1/692)	37	.89 (1/112)

[a] This number is based on an estimate of a 95% detection rate of MLIV carriers.
[b] 95% CI = .52%–1.39%.
[c] 95% CI = .40%–1.17%.

(Dong et al. 2001). Variation in the frequency of MLIV carriers between the United States and Israel thus provides further evidence that Ashkenazi subpopulations have not reached equilibrium.

A more recent origin for the two AJ MLIV founder mutations would explain why the frequency of carriers for MLIV is not as high as the frequencies of other mutations that are common in the AJ population, including some that originated in or were introduced into the European Jewish population >1,000 years ago (Diaz et al. 2000). The carrier frequencies of these disorders range from 1/18, for Gaucher disease (Beutler and Grabowski 2000), to 1/107, for Bloom syndrome (Li et al. 1998). The observed frequency of MLIV carriers within a large cohort of AJ individuals from the New York metropolitan area is 1/127, slightly lower than the frequency of disorders for which carrier screening is currently available. However, in light of the neurologic severity of the MLIV phenotype, for which there is no available treatment, and the fact that the two mutations account for >95% of carriers, carrier screening for the two mutations should be considered for this population.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Genbank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *Homo sapiens MCOLN1,* complete coding sequence [accession number AF287270])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for MLIV [MIM 252650] and mucolipin-1 [MIM 605248])

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13201 ggccacccag gaattttcca agtaacgact cggagtctcc gggattccta tctcccggcc
13261 cccgaatttc
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Jan 30 2006 12 09 03

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Sun, et al.

Title: METHODS AND COMPOSITIONS FOR THE DETECTION OF MUCOLIPIDOSIS IV MUTATIONS

Appl. No.: 10/754,446

Filing Date: January 9, 2004

Examiner: Kapushoc, Stephen Thomas

Art Unit: 1634

Confirmation Number: 7990

CERTIFICATE OF MAILING
I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date below.

Marisol Moretto
(Printed Name)

(Signature)

August 10, 2006
(Date of Deposit)

DECLARATION OF WEIMAN SUN, PH.D UNDER 37 C.F.R §1.132

Commissioner for Patents
PO Box 1450
Alexandria, Virginia 22313-1450

Sir:

I, Weiman Sun, Ph.D. hereby declare as follows:

1. I attended the University of California, Davis where I received a Ph.D degree in Pharmacology and Toxicology in 1994.

2. I was a postdoctoral fellow at UCLA Intercampus Medical Genetics Training program for over 3 years. Then, I served as the director of the molecular genetics laboratory (The Genetics Center) and the manager of the molecular pathology lab (UC Irvine) before joining Quest Diagnostics as an associate scientific director and later the R&D manager of the Molecular Genetics Department. I am currently the Scientific Director of Molecular Genetics at Quest Diagnostics Nichols Institute, Assignee of the patent application.

3. In total, I have worked more than 12 years in the field of nucleic acid diagnostic assays. I am the author or co-author of more than 29 published scientific articles and

many abstracts in this field. A brief summary of my accomplishments and a recent copy of my Curriculum Vitae is attached as APPENDIX 1.

4. I am a co-inventor of the above identified patent application. I understand that the Examiner has rejected the claims as being obvious over a combination of prior art references. According to the Examiner, one of ordinary skill would have been motivated to take various features from these references and combine them to obtain the claimed invention and that there would have been a reasonable expectation of success in achieving the combination. The Examiner bases this conclusion on the belief that because the entire sequence of MCOLN1 is known, it would have required nothing more than ordinary skill to develop a MCOLN1 real time assay using any particular primer or probe sequence that can be derived from the MCOLN1 genome. In my opinion, this rationale is flawed. My own experience from 12 years of assay development and from others who have published in this field leads me to conclude that a successful nucleic acid based assay is very much dependent on the particular primers and probe sequences chosen and that there is no way to predict in advance whether particular sequences would be effective.

5. It is my experience that real time amplification assays such as the TaqMan assay are more complex than standard PCR assays because the primers and the probe in a TaqMan format must be able to function together at the same time since the probe must bind to amplified product as it is extended from the primers. The probe in standard PCR is applied after the PCR has been completed and amplicons generated. Because of the more stringent working requirements, primers pairs and probes that might work acceptably in standard PCR are more likely to fail or perform poorly in the TaqMan format. I conclude from this that there would not have been a reasonable expectation of success for combining the probes of Edelmann et al. from standard PCR assays, for use in a TaqMan style PCR assay such as described in Doll et al. as asserted by the Examiner.

6. Unlike mutation analysis methods that analyze amplification products from conventional PCR reactions, our amplification primers as well as mutation detection (allelic-discrimination) probes need to work together in the same homogeneous condition balancing the need for high amplification efficiency and the capability in distinguishing the

7. As described in Section 5.1 (Primer and probe design guidelines) in Real-Time PCR (Advanced Methods Series) (Dorak MT, Oxford: Taylor & Francis, 2006), real-time PCR has different needs than conventional PCR. Different considerations for real-time PCR include, for example, primer melting temperature, probe melting temperature, amplification product length, and placement of primers and probes.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the captioned patent application or any patent issued therefrom.

8. 9. 2006

Date

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Birth Date: June 7, 1965
Marital Status: Married
Citizenship: U.S.A.

EDUCATION

Pharmacology and Toxicology, University of California, Davis. 09/89 - 07/94
Ph.D., September 1994.
Advisor: Dr. Jerold A. Last

Basic Medicine, Shanghai Medical University, 09/82 - 06/88
MD, June 1988

PROFESSIONAL EXPERIENCE

Scientific Director, Molecular Genetics Department, Quest Diagnostics Incorporated, 2002-present
1. Responsible for overall design, performance and maintenance, as well as technical and clinical validation of all testing in Molecular Genetics
2. Manage R&D portfolio design/planning, staffs and projects
3. Review clinical information and laboratory results for various genetic tests to provide final diagnosis and interpretations
4. Provide consultation to physicians and genetic counselors on genetic testing

Research and Development Manager, Molecular Genetics Department, Quest Diagnostics Incorporated, 2001
1. Manage R&D staffs (senior scientists and research associates) and projects
2. Review clinical information and laboratory results for various genetic tests to provide final diagnosis and interpretations
3. Provide consultation to physicians and genetic counselors on genetic testing
4. Participate in QA&QC programs

Associate Scientific Director of Molecular Genetics Department, Quest Diagnostics Incorporated, 2000
1. Review clinical information and laboratory results for various genetic tests to provide final diagnosis and interpretations
2. Provide consultation to physicians and genetic counselors on genetic testing
3. Manage R&D staffs and projects
4. Participate in QA&QC programs

Manager of Diagnostic Molecular Pathology Laboratory, UC Irvine, 1999 - 2000
1. Set up the molecular diagnostics laboratory, including purchasing equipment and supplies, designing laboratory layout and developing/implementing quality control program
2. Developed and validated molecular diagnostic assays, including thrombophilia variant analysis (Factor V Leiden, prothrombin and MTHFR), Fragile X syndrome, HFE genotype analysis (C282Y and H63D) and parentage/genetic identity testing
3. Managed the laboratory according to CLIA/CAP guideline, troubleshot technical problems and trained technologist and participated in proficiency/interlab exchange testing
4. Written standard operational procedures of the laboratory and pamphlets for test information
5. Designed and developed a PC database for general laboratory management

Director of Molecular Genetics Laboratory, Genetics Center, 1998
1. Set up the molecular diagnostics laboratory, including purchasing equipment and supplies, designing laboratory layout, developing quality assurance protocols and training technologists.

2. Developed and validated molecular diagnostic assays, including cystic fibrosis (analysis for 33 mutations and 1 polymorphism), Fragile X syndrome, Ashkenazi Jewish genetic diseases panel (Canavan, CF, Gaucher and Tay-Sachs), genetic identity tests, myotonic dystrophy, BCR-ABL translocation (RNA-based), Prader-Willi/Angelman syndromes (methylation PCR) and sex chromosome analysis.
3. Supervised daily laboratory operation, troubleshot technical problems, reviewed and signed-out diagnostic cases.
4. Developed report forms on molecular genetic tests; given presentations and lectures to clinicians and written newsletter articles on molecular tests.

<u>Postdoctoral Work</u>, UCLA, 1994 – 1997

1. Identified the molecular defect in aryl hydrocarbon receptor protein expressed by a mutant strain of a mouse hepatoma cell line. Characterized the effect of a single point mutation in the coding region of the mutant protein on its functionality in protein (ARNT)-heterodimerization, DNA binding and transactivation.
2. Studied possible human genetic polymorphisms in inducibility of cytochrome *P-4501A1* (*Cyp1A1*) gene by using peripheral blood mononuclear cells collected from healthy volunteers and placenta tissue. Developed competitive rt-PCR assay to accurately quantify the expression of *CYP1A1* gene as well as its regulators. Developed methodology to amplify and clone full-length coding cDNA of the relevant genes for *in vitro* expression and functional analysis.
3. Applied representational difference analysis (RDA) technique to study the tumor-promotion activity of TCDD by isolating and analyzing the affected genes.
4. Received training in applying DNA technology to clinical diagnostics. Developed a simple PCR-based procedure to test for Familial Haemochromatosis and a RNA-based assay to detect chromosome translocation.

<u>Graduate Study</u>, UC Davis, 1989 - 1994

1. Studied the acute effects of airborne toxicants (ozone and tobacco smoke) on human airway epithelial cells *in vitro*. Defined cytotoxicities of air pollutants with various viability assays. Analyzed changes in protein synthesis patterns in injured cells. Characterized the induced expression of a 45kDa protein by ozone and environmental tobacco smoke (ETS). Generated and purified internal peptides from the 45kDa protein for N-terminal micro-sequencing analysis.
2. Tested the importance of inflammation in enhancing the direct toxic effects of ozone and nitrogen dioxide mixture to rat lungs *in vivo*.
3. Teaching Assistant (1992) for Department of Environmental Toxicology in *Air Pollution and Inhalation Toxicology*. Participated in designing and grading homework and exam questions, helped students during office hours, coordinated guest lecturers and provided demonstration facilities.

<u>Research Assistant</u>, Shanghai Medical University, 1982 - 1988

Used immunohistochemistry methods to study the distribution and expression of desmin in human fetal organs and tissues, as well as poorly differentiated sarcomas; Professor Yue-er Zhang, Department of Pathology

PUBLICATIONS

Last, J.A., Wu, R., Jin, C., Gelzleichter, T., **Sun, W.**, Armstrong, L.C. (1990) Particle-cell interactions – lung fibrogenesis. Journal of Aerosol Medicine 3(S1): S61-S74

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(Ph.D thesis)

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Sun, W., Zhang, J., and Hankinson, O. (1997) A mutation in the aryl hydrocarbon receptor (AHR) in a cultured mammalian cell line identifies a novel region of AHR that affects DNA binding. Journal of Biological Chemistry 272(50): 31845-54

Antilla, S., Lei, X., Elovaara, E., Karjalainen, A., **Sun, W.**, Vainio, H., and Hankinson, O. (2000) An uncommon phenotype of poor inducilbility of CYP1A1 in human lung is not ascribable to polymorphisms in the *AHR*, *ARNT*, or *CYP1A1* genes. Pharmacogenetics 10: 1-11

Roth MD, Marques-Magallanes JA, Yuan M, **Sun W**, Tashkin DP, Hankinson O. (2001) Induction and Regulation of the Carcinogen-Metabolizing Enzume, CYP1A1, by Marijuana Smoke and Δ^9-Tetrahydrocannabinol. American Journal of Respiratory Cell and Molecular Biology. 24(3):339-44.

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Strom, CS, Huang, S, Chen, C, Buller, A, Peng, M, Quan, F, Redman, R, **Sun, W**. (2003) Extensive sequencing of the Cystic Fibrosis Transmembrane Regulator gene: Assay validation and unexpected benefits of developing a comprehensive test. Genetics in Medicine, 5(1): 9-14

French C, Li C, Strom C, **Sun W**, Van Atta R, Gonzalez B, Wood M. (2004) Detection of the factor V Leiden mutation by a modified photo-cross-linking oligonucleotide hybridization assay. Clinical Chemistry 50(2):296-305. Epub 2003 Dec 04

Strom CM, Clark DD, Hantash FM, Rea L, Anderson B, Maul D, Huang D, Traul D, Chen Tubman C, Garcia R, Hess PP, Wang H, Crossley B, Woodruff E, Chen R, Killeen M, **Sun W**, Beer J, Avens H, Polisky B, Jenison RD. (2004) Direct visualization of cystic fibrosis transmembrane regulator mutations in the clinical laboratory setting. Clinical Chemistry, 50(5):836-45. Epub 2004 Mar 09.

Strom CM, Crossley B, Redman JB, Buller A, Quan F, Peng M, McGinnis M, **Sun W.** (2004) Cystic fibrosis screening: lessons learned from the first 320,000 patients. Genetics in Medicine. 6(3):136-40

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Strom CM, Janeszco R, Quan F, Wang SB, Buller A, McGinniss M, **Sun W.** (2006) Technical validation of a tm biosciences luminex-based multiplex assay for detecting the american college of medical genetics recommended cystic fibrosis mutation panel. Journal of Molecular Diagnostics. 8(3):371-5.

ABSTACTS AND MEETING PRESENTATIONS

Sun, W., Chau, D., Krolewski, J., Selsted, M., Kagan, R., and Fenwick, R. (2000) A multiplex allele-specific amplification assay detects two common variants in the human HFE gene associated with hereditary hemochromatosis. (presented at the AMP annual meeting)

Sun, W., Buller, A, Huynh, N., Huang, S., Vu, M., Avila, L., Chen, C., Bower, B., York, S., Entwistle, T., and Strom, C. (2001) Genotype frequencies of C282Y and H63D alleles in the hemochromatosis (HFE) gene from patient samples submitted to a reference laboratory for HFE genotyping. (presented at the 2001 ASHG

annual meeting)

Anderson, B., **Sun, W.**, Buller, A., Huang, S., Hantash, F., Wang, S., Entwistle, T., Strom, CS. (2001) A novel technique to accurately assess engraftment percentages. (presented at 2002 ACMG annual meeting)

Qu, K., **Sun, W.**, Huynh, N., Strom, C., Popov, J., and Sferruzza, A. (2002) Simultaneous Detection of Methylenetetrahydrofolate Reductase Mutations C677T and A1298C by Fluorescent Restriction Fragment Length Polymorphism. (Presented at the 2002 AACC annual meeting)

Sun W, Buller A, Fenwick R, Hantash F, Huang S, Peng M, Redman J, and Strom CS (2002) Spectrum of Mutations Detected in the RET Proto-oncogene Associated with Multiple Endocrine Neoplasia Type 2 (Presented at 2002 ASHG annual meeting)

Strom C, **Sun W**, Zoleikhaeian M, Fenwick R, York M, Entwistle T. (2002) Comparison of SNP Detection Methods and Instrumentation Platforms (Presented at 2002 ASHG annual meeting)

Sun W, Redman J, Wallenstein R, McCarrier J, Lee D, Buller A, McGinniss M, Quan F, Huang S, and Strom C. (2003) Comprehensive Sequence Analysis Ruled out Disease Association with the CFTR Gene in a Family with Atypical CF Presentations (Presented at 2003 ASHG annual meeting)

Anderson B, **Sun W**, Redman J, Buller A, McGinniss M, Quan F, and Strom C (2004) CFTR Gene Haplotype Determines the Phenotype Associated with the 5T Variant (Presented at 2004 ASHG annual meeting)

Sun W, Potts S, Peng M, Buller A, McGinniss M, Quan F, Taylor J, and Strom C (2005) Novel Mutations and Variants Identified in the MECP2 Gene (Presented at 2005 ASHG annual meeting)

(Only presentations with my being a 1st or 2nd author are included)

PROFESSIONAL BOARD CERTIFICATION

American Board of Medical Genetics (Clinical Molecular Genetics,)
New York State Certification of Qualification for Laboratory Director (DNA)
Clinical Genetic Molecular Biologist by the State of California
Clinical Genetic Molecular Biologist Scientist by the State of California
Clinical Laboratory Scientist (Molecular Biology) by National Certification Agency (NCA)

HONORS

- Regents Fellowship, UCDavis, 1990-1991
- Nonresident Tuition Fellowship, UCDavis, 1989, 1991-1993
- GSA Travel Grant, UCDavis, 1993
- Research Training Grant Fellowship, American Lung Association, 1995-1996
- Intercampus Medical Genetics Training Grant, NIH, 1996 (Jan.) – 1997 (Dec.)

PROFESSIONAL AFFILIATIONS

American Society of Human Genetics (ASHG)
Association for Molecular Pathology (AMP)
American Association for the Advancement of Science (AAAS)
Phi Sigma Biological Honor Society (elected and declined)

REFERENCES

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